

**Interventions in WNT signaling to induce cardiomyocyte proliferation:
crosstalk with other pathways**

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AKT: protein kinase B; APC: adenomatous polyposis coli protein; CK1: casein kinase-1; CM: cardiomyocyte; DKK: Dickkopf; Dvl: Dishevelled; FSTL-1: follistatin like-1; GH: growth hormone; GSK3: glycogen synthase kinase-3; IGF: insulin-like growth factor; LAD: left anterior descending coronary artery; LRP: lipoprotein-related receptor protein; MAPK: mitogen-activate protein kinase; MI: myocardial infarction; NRG1: neuregulin-1; PHH3: phosphorylated histone-3; PI3K: Phosphoinositide 3-Kinase; sFRP: soluble Frizzled-related protein; TAZ: Transcriptional co-activator with PDX binding motif; TCF/LEF: T-cell factor/lymphoid enhancer-binding factor; WNT: Wingless/Int-1 protein; YAP: Yes-associated protein

Abstract

Myocardial infarction is a frequent cardiovascular event and a major cause for cardiomyocyte loss. In adult mammals, cardiomyocytes are traditionally considered to be terminally differentiated cells, unable to proliferate. Therefore, the wound healing response in the infarct area typically yields scar tissue, rather than newly formed cardiomyocytes. In the last decade, however, several lines of evidence have challenged the lack of proliferative capacity of the differentiated cardiomyocyte: studies in zebrafish and neonatal mammals have convincingly demonstrated the regenerative capacity of cardiomyocytes. Moreover, multiple signaling pathways have been identified in these models which – when activated in adult mammalian cardiomyocytes- can reactivate the cell cycle in these cells. However, cardiomyocytes frequently exit the cell cycle before symmetric division into daughter cells, leading to polyploidy and multinucleation. Now that there is more insight into the reactivation of the cell cycle machinery, other prerequisites for successful symmetric division of cardiomyocytes, such as the control of sarcomere disassembly to allow cytokinesis, require more investigation. This review aims to discuss the signaling pathways involved in cardiomyocyte proliferation, with a specific focus on WNT signaling. Comparing the conflicting results from *in vitro* and *in vivo* studies on this pathway illustrates that the interaction with other cells and structures around the infarct is likely to be essential to determine the outcome of these interventions. The extensive crosstalk with other pathways, implicated in cardiomyocyte proliferation, calls for the identification of nodal points in the cell signaling, before cardiomyocyte proliferation can be moved forward towards clinical application as a cure of cardiac disease.

Significance statement

Evidence is mounting that proliferation of pre-existing cardiomyocytes can be stimulated to repair injury of the heart. In this review article, an overview is provided of the different signaling pathways implicated in cardiomyocyte proliferation with emphasis on WNT signaling, crosstalk between the pathways and controversial results obtained *in vitro* and *in vivo*.

Introduction

Diseases of the heart are among the most common causes of morbidity and mortality in man. These diseases include disturbances in cardiac rhythm, contractility and output and, despite their highly variable etiology, all result in a loss of pump function to a point where the cardiac output is insufficient to meet the needs of the body. Loss of cardiomyocytes (CMs) generally is considered to be a common contributing factor to the impaired pump function. This becomes particularly apparent after myocardial infarction (MI), where many CMs in the infarct area are lost due to an acute interruption of the blood supply.

Replenishing of the lost CMs has for many years been proposed as the Holy Grail to repair the damaged heart. To achieve this, the injection of (stem) cells, derived from multiple sources has been thoroughly explored, particularly in a context of MI. Unfortunately, the beneficial effects of cell delivery to the infarcted heart have turned out to be disappointing in a clinical setting, because of the poor engraftment of the cells in the cardiac tissue as well as issues with immaturity and induction of arrhythmias (Braunwald, 2018).

More recently, the stimulation of endogenous repair mechanisms has been brought forward as a novel approach to repairing cardiac damage. Evidence is mounting that the dogma that adult CMs cannot proliferate may not be true (Bergmann, 2019; Bergmann et al., 2009). Moreover, an increasing number of studies has shown that activation of a variety of signaling pathways can in fact reactivate the cell cycle in CMs past the point of cytokinesis, yielding symmetric division into two daughter cells (Ponnusamy et al., 2017). In this review, we provide an overview of studies where interventions in WNT signaling result in CM proliferation and cardiac regeneration. These findings will be placed in a context of several other signaling pathways, implicated in CM proliferation, in order to identify crosstalk between the pathways and nodal points that are critical in its control.

Wound healing response following myocardial infarction

Cardiac injury following MI evokes a wound healing response very similar to skin wound healing, as previously described in detail (Cleutjens et al., 1999). Initially, polymorphonuclear neutrophils invade the infarct area somewhere between 6 and 12 hours after the onset of the ischemia, where they commence with the clearing of the necrotic debris. The necrotic tissue is replaced by granulation tissue, rich in (myo)fibroblasts, macrophages and immature blood vessels embedded in newly formed and relatively unorganized extracellular matrix. In the weeks following the ischemic event, the granulation tissue matures: the number of blood vessels and myofibroblasts decrease, the macrophages disappear and the amount of deposited extracellular matrix increases to form a scar (van den Borne et al., 2010). Remarkably, however, myofibroblasts remain present in a well-healed cardiac scar to maintain the integrity of the matrix and prevent excessive dilatation of the scar, although the presence of myofibroblasts in the viable myocardium can contribute to stiffness and impairs diastolic function of the ventricles (Hermans et al., 2016).

In the past decade, it has become increasingly clear that the epicardium plays a pivotal role during cardiac development and repair. Using lineage tracing techniques, epicardial cells have been shown to contribute to vascular smooth muscle cells, pericytes and fibroblasts in the injured heart. Moreover, the epicardium has been identified as a rich source of signaling molecules that can modulate the wound healing response and may even stimulate proliferation of CMs around the infarct area. In contrast, the contribution of epicardial cells to the pool of CMs is considered to be minimal (Cao and Poss, 2018). This observation illustrates that the wound healing post-MI involves complex crosstalk between different cells and structures in and around the infarct area. In this review, the question will be addressed whether interventions in this crosstalk can stimulate resident CMs in the border zone to proliferate, thereby reducing the size of the infarct and improving the cardiac function that was impaired by the injury.

Evidence for cardiomyocyte proliferation

Adult mammalian CMs are generally viewed as terminally differentiated, post-mitotic cells which have lost their ability to proliferate during maturation (Zak, 1973). Instead, the typical response of the heart to an increased workload or injury is through cardiac hypertrophy, where an enlargement of the CMs, rather than a higher number of these cells, is responsible for the adaptation of the heart (Gunthel et al., 2018). The contribution of resident cardiac progenitor cells to cardiac regeneration has been proposed in the past, but is now considered irrelevant (Eschenhagen et al., 2017). Instead, several lines of evidence have recently challenged the dogma that CM proliferation is completely absent in the adult heart and that these cells cannot be directed towards a proliferative state to repair the injured heart, as schematically summarized in Fig. 1.

First of all, CMs of all vertebrates –including humans- show extensive proliferation during the looping phase of the developing heart tube, leading to the ballooning of the ventricles and formation of the four-chambered heart. Moreover, the heart tube is formed by cells migrating from the primary and secondary heart field, and these cells temporarily stop proliferating when the heart tube is formed but then commence their proliferation (Gunthel et al., 2018). Taken together, these findings indicate that in embryonal vertebrates, non-proliferating CMs can re-enter the cell cycle, provided that they receive the appropriate signals from their environment. In this context, it is relevant to note that CM regeneration in response to cardiac injury has also been observed in fetal and neonatal mice (Lam and Sadek, 2018). However, injuries inflicted to mouse hearts later than one week after birth lead to scarring rather than CM proliferation, suggesting that the regenerative capacity of the heart is lost shortly after birth (Foglia and Poss, 2016). The post-natal rise in oxygen content has been proposed to induce the cell cycle arrest of the CMs (Puente et al., 2014). This concept is supported by the observation that gradual exposure to severe hypoxia can re-active mitosis in the CMs in the adult mouse heart (Nakada et al., 2017).

A second line of evidence for proliferation of adult CMs was found in teleost fish (e.g. zebrafish) and some urodele amphibians (e.g. red-spotted newt). Upon resection of the ventricular apex, these animals show full regeneration of cardiac tissue that is populated with CMs that are functionally coupled to the remaining myocardium (Poss et al., 2002; Witman et al., 2011). Similar results were obtained in a zebrafish cryoinjury model, although in this model the damaged CMs initially were replaced by scar tissue before the area was repopulated with CMs (Gonzalez-Rosa et al., 2011).

Lineage tracing experiments have shown that these newly-formed CMs are actually derived from pre-existing, differentiated CMs rather than from stem or progenitor cells (Jopling et al., 2010).

CM proliferation is not restricted to animal models such as zebrafish or neonatal mice, but has also been reported to occur in adult humans. In a landmark study, Bergmann et al. made use of the peak in the atmospheric ^{14}C concentration due to aboveground nuclear bomb testing in the 1950's, which dropped dramatically after banning these practices in 1963. This peak allowed them to carbon-date the DNA in left ventricular myocytes. The authors concluded that there was a 1% CM turnover in young adults, which gradually declined to ~0.5% in 75 year-olds, with an estimated 45% of CMs being generated after birth in a 50 year-old person (Bergmann et al., 2009). At present, it is unclear whether cardiac injury can augment CM proliferation in adult humans. It is obvious, however, that this response is inadequate to completely repair cardiac damage as e.g. observed in adult zebrafish.

A complicating factor in the study of CM proliferation is the tendency of these cells to develop polyploidy and multinucleation. Multinucleation is a common event in CMs after birth, with ~90% binucleation in adult rodents and an increase of ploidy with age in humans. This suggests that adult CMs, although capable of entering the cell cycle, infrequently complete this process with cytokinesis to yield two mononucleated daughter cells (Ponnusamy et al., 2017). Multinucleation and polyploidy are indicators of permanent cell cycle withdrawal, but at present it is unclear whether these processes actively inhibit cell cycle completion by cytokinesis (Yuan and Braun, 2017) or whether an aberrant microtubule distribution in the CM leads to binucleation rather than proper cell division (Leone et al., 2018).

Current detection techniques of cell proliferation, such as detection of mitotic figures, incorporation of labeled DNA building blocks (e.g. BrdU) or immunohistochemical detection of cell cycle markers (e.g. Ki67 or Aurora B kinase), insufficiently discriminate true CM proliferation from multinucleation events (Ponnusamy et al., 2017). It is important to note that degradation of the contractile apparatus of the CM is required for the completion of the cell cycle with cytokinesis to yield two daughter cells (Jopling et al., 2010; Szibor et al., 2014). Novel methods have recently been proposed to identify true CM proliferation, including midbody positioning combined with a correct spacing of the daughter nuclei (Hesse et al., 2018), CM-specific expression of Fluorescence ubiquitination-based Cell Cycle Indicator (FUCCI, (Alvarez et al., 2019)) and Multiple-isotope Mass Spectrometry (MIMS (Senyo et al., 2013)). Using the latter technique, Senyo et al. calculated that ~3.2% of the CMs around the infarct area had

undergone division at 8 weeks post-MI in mice (Senyo et al., 2013), indicating that proliferation of these cells actually can occur in response to cardiac ischemia.

Molecular control of cardiomyocyte proliferation

The intriguing capacity of zebrafish for cardiac regeneration has stimulated investigations into the molecular pathways inducing CM proliferation in the area of injury. There is a growing body of evidence that interventions in WNT signaling can induce cardiac regeneration. However, WNT signaling does not operate in isolation, since multiple signaling pathways have been implicated in the regenerative response of zebrafish. Follistatin-like 1 (FSTL-1), Hippo/Yap/TAZ, neuregulin, and Notch signaling appear to be the most important in this respect. These pathways have been investigated in mammalian models of cardiac remodeling, mostly MI. In the following paragraphs, an overview of the literature on WNT signaling in cardiac regeneration will be presented. Subsequently, evidence for the involvement of FSTL-1, Hippo/Yap/TAZ, neuregulin, and Notch signaling in cardiac regeneration will be discussed briefly. The potential nodal points where these pathways can interact will be discussed in the next paragraph.

WNT signaling

WNT signaling plays an essential role in developmental biology of a wide variety of animals. A total of 19 different WNT proteins has been identified in mammals, typically 350-400 aa in size. WNT proteins undergo extensive posttranslational modification to become biologically active: N-linked glycosylation and lipid modification at two distinct sites in the protein. Palmitoleoylation by an enzyme called Porcupine is essential for transportation of WNT proteins to the plasma membrane, where Wntless (Wls) is responsible for the secretion of WNTs in order to interact with neighboring cells (MacDonald et al., 2009).

WNT ligands interact with a complex of receptor proteins in order to activate their intracellular signaling pathways. Originally, proteins from the Frizzled family were identified as receptors for WNT proteins. In the meantime, the involvement of the co-receptors LRP5 and -6 has been firmly established (Schulte, 2010). Several classes of secreted modulators of WNT signaling have been identified, including the members of the Dickkopf (DKK) family. In mammals, this family consists of 4 members, which –with the exception of DKK3– can inhibit β -catenin-mediated WNT signaling by binding to the LRPs with high affinity (Cruciat and Niehrs, 2013).

Upon binding to the receptor complex, WNT proteins can activate distinct signaling pathways. The most extensively studied pathway involves β -catenin as its second messenger, and is frequently

referred to as 'canonical' WNT signaling. In the inactive state, β -catenin is phosphorylated by the kinases Casein Kinase-1 (CK1) and Glycogen Synthase Kinase-3 β (GSK3 β), two components of the so-called destruction complex located in the cytoplasm. This phosphorylation is the first step in the degradation of β -catenin. Activation of the Frizzled/LRP receptor complex by WNT, however, induces the translocation of the destruction complex to the plasma membrane where it becomes inactive. The net result of this is an increase in the concentration of cytoplasmic β -catenin, which can subsequently migrate to the nucleus. There, β -catenin can associate with transcription factors of the TCF/LEF family and induce gene transcription (MacDonald et al., 2009). Next to β -catenin-mediated signaling, Planar Cell Polarity signaling and signaling via Ca^{2+} have been described (Schulte, 2010), but their role in cardiac regeneration is only sparsely studied.

In vertebrates, the heart develops from two sets of precursor cells, the first and secondary heart field. After formation of the linear heart tube by cells from the first heart field, mesodermal cells from the secondary heart field migrate towards the tube. The proliferation of the secondary heart field cells is dependent on activation of WNT/ β -catenin signaling (Gunthel et al., 2018). WNT signaling has been shown to form a gradient, which correlates positively with CM proliferation over the ventricular wall, and activation of WNT/ β -catenin signaling by deletion of APC led to ventricular hyperplasia (Ye et al., 2015). The role of WNT signaling in the development of CMs from embryonic stem cells was shown to be biphasic, depending on the developmental stage: activation of WNT/ β -catenin signaling is required for mesodermal specification of embryonic stem cells, but further CM differentiation requires the inhibition of WNT/ β -catenin signaling (Ozhan and Weidinger, 2015). This underscores the complex role of Wnt/ β -catenin signaling in the differentiation of CMs.

Activation of WNT signaling during infarct healing has not only been demonstrated by studying the changes in expression of its components (Barandon et al., 2003) but also by the use of β -catenin sensitive reporter constructs. Using a LacZ reporter mouse under the control of the Axin2 promotor, Oerlemans et al. reported extensive labeling of endothelial cells, leucocytes, (myo)fibroblasts and Sca-positive cells (Oerlemans et al., 2010). Similar results were obtained in mice where LacZ expression was driven by a TCF/LEF-responsive promotor (Aisagbonhi et al., 2011). AAV9-mediated transduction of a chimeric probe consisting of human ferritin heavy chain controlled by the TCF/LEF promotor into infarcted rat hearts allowed the detection of WNT/ β -catenin signaling in the border zone at 4 weeks

post-MI by magnetic resonance imaging (Matteucci et al., 2016). These studies provide strong support for the activation of WNT signaling in the border zone of the infarct, albeit that the nature of the reporter constructs only allows the detection of WNT/ β -catenin signaling; the status of the activation of the other WNT signaling pathways after MI is at present less clear.

In vitro modulation of WNT signaling

The importance of WNT/ β -catenin signaling in embryonic heart development has stimulated many researchers to study the effects of interventions in this pathway on the proliferation and differentiation of various precursor cells towards CMs. Activation of WNT/ β -catenin signaling in embryonic CMs, isolated from the first and secondary heart field, showed robust stimulation of the proliferation of these cells (Buikema et al., 2013). Using a chemical screen, Uosaki et al. identified the GSK3 β -inhibitor BIO as a potent inducer of cell cycle markers in mouse pluripotent stem cell-derived CMs (Uosaki et al., 2013). In matured ES-cell-derived CMs, Fan et al. observed that activation of WNT/ β -catenin signaling, either by inhibition of GSK3 β or by liberating the N-cadherin-bound β -catenin in the cytoplasm. This resulted in increased incorporation of BrdU and increased expression of multiple cyclin genes, suggesting the induction of cell proliferation (Fan et al., 2018).

In several other studies, CMs from neonatal rats were used to study the effect of the activation of WNT/ β -catenin signaling on their proliferation. Activation of WNT signaling via GSK3 β inhibition with BIO (5 μ M) resulted in a 10-fold increase in BrdU incorporation and a 5-fold increase in phospho-histone 3 (pHH3)-positive cells (Tseng et al., 2006). These results were confirmed by Kim et al. (Kim et al., 2016), who showed that BIO not only induced BrdU incorporation in cultured neonatal rat CMs but also reduced BrdU incorporation in rat neonatal cardiac fibroblasts. Increased proliferation was also observed in cultured human atrial CMs treated with the GSK3 β inhibitor CHIR99021, albeit that the increase in proliferating CMs was modest (control: $1.83 \pm 0.01\%$; CHIR: $2.67 \pm 0.08\%$); in this study, inhibition of WNT/ β -catenin signaling with IWR-1 resulted in a ~35% reduction in CM proliferation (Wang et al., 2016). Adenoviral-mediated overexpression of β -catenin in neonatal rat CM, however, showed an initiation of the cell cycle; this did not result in an increased number of CMs but augmented CM hypertrophy and the number of binucleated cells (Hahn et al., 2006). These observations suggest that, although WNT signaling is capable of initiating the cell cycle, other factors are needed to complete the process with cytokinesis.

In vivo responses to modulation of WNT signaling

In a growing number of studies, the effects of modulation of WNT signaling has been studied in animal models of MI. Here, we will discuss the experimental evidence, starting with interventions at the level of WNT ligand and its extracellular scavenging by sFRPs, and followed by interventions at the level of the receptor complex, intracellular signaling and modulation of β -catenin-mediated gene transcription

Interventions at the level of WNT

Stimulation of the WNT signaling pathway by increasing the amount of WNT protein in the infarct area has been tested in several studies. The direct injection of WNT3a in the border zone of the infarct resulted in an increased infarct area and reduced cardiac function (FS) at one week post-MI in mice (Oikonomopoulos et al., 2011). In contrast, beneficial effects on infarct size were reported in mice overexpressing WNT10b in CMs or WNT11 via AAV9-mediated gene transfer. In both studies, this was accompanied by improved pump function of the heart. The proposed mechanisms were an increased neovascularization with reduced fibrosis in the WNT10B overexpression study (Paik et al., 2015) and reduced incorporation of bone marrow-derived inflammatory cells in the AAV9-WNT11 study (Morishita et al., 2016), respectively. It is unclear how the differences between the three studies can be explained, because WNT3a, -10b and -11 all have been reported to stimulate β -catenin-mediated WNT signaling (MacDonald et al., 2009).

An alternative way of intervening in WNT signaling is to inhibit crucial steps in its synthesis or transport to the extracellular space. Attenuation of WNT secretion in macrophages by selectively inactivating the expression of the WNT transporter protein Wntless reduced adverse remodeling and improved cardiac function at 30 days post-MI. This was accompanied by an M2 polarization of the macrophages, which reduces inflammation and promotes angiogenesis and repair (Palevski et al., 2017). Palmitoylation of WNTs is essential for their biological function (Harterink and Korswagen, 2012) and administration of inhibitors of Porcupine, the enzyme responsible for the attachment of the lipid moiety to WNT proteins, results in the inhibition of the secretion of all WNTs that are expressed in the cell. WNT inhibition with the porcupine inhibitor GNF-6231, administered intravenously after induction of MI, reduced the infarct size and attenuated the decline in cardiac function at 30 days post-MI. Histologically, fewer apoptotic CMs were detected and the number of myofibroblasts in the border zone was reduced (Bastakoty and

Young, 2016). A similar reduction in infarct size with a concomitant improvement in cardiac function was reported in mice treated systemically with the porcupine inhibitor WNT-974 from week 1-10 after MI induction (Moon et al., 2017). The authors conclude, however, that an anti-fibrotic effect involving Collagen-VI was responsible for the beneficial effect on infarct healing of WNT-974. In contrast to the studies with porcupine inhibitors mentioned above, Yang et al. not only demonstrated reduced infarct size and improved function, but also a significant increase in newly formed CMs in the border zone of mice treated with the porcupine inhibitor CGX1321 for 4 weeks post-MI (Yang et al., 2017). The total number of EdU-positive mononucleated adult CMs was found to be significantly upregulated, indicative for induction of CM proliferation by the porcupine inhibitor. Taken together, these studies consistently show that pharmacological inhibition of WNT secretion in the infarct area has beneficial effects on infarct size and cardiac function *in vivo*.

Modulation at the receptor level

Activation of WNT signaling has been convincingly demonstrated during the wound healing process following MI (Foulquier et al., 2018). Although several studies have demonstrated the upregulation of Frizzled proteins during infarct healing (Barandon et al., 2003; Blankesteyn et al., 1997), interventions targeting Frizzled proteins in a model of cardiac ischemia are scarce. Using a 13 amino acid peptide fragment of WNT5a, named UM206, we could demonstrate a reduced infarct size in mice (Laeremans et al., 2011) and swine (Uitterdijk et al., 2016). In mice, this was accompanied by an improved cardiac function and prevention of mortality post-MI. The mechanism of action of this peptide, however, is obscured by its location in the ligand-receptor complex (Janda et al., 2012), making a direct interaction of the peptide with the binding interfaces of WNT and Frizzled is unlikely.

The role of the LRP-coreceptors in infarct healing has been studied more extensively. Induction of MI in mice deficient in LRP5 resulted in an increase in infarct size (Borrell-Pages et al., 2016), but no change in infarct area or cardiac function as observed when LRP5 was specifically deleted in CMs (Wo et al., 2016). In several studies, the effect of DKK proteins, endogenous antagonists of LRP proteins, on infarct healing was addressed. Injection of DKK1 in the border zone of the infarct area in mice did not change infarct size or function (Wo et al., 2016). In contrast, DKK2 injection reduced the size of the infarct and improved cardiac function in rats at 1 week post-MI, which was attributed by the authors to a positive effect on angiogenesis (Min et al., 2011). Beneficial effects on infarct healing

were observed in mice overexpressing DKK3 in a CM-specific manner, although the authors proposed a cardioprotective mechanism via ASK1-JNK/p38 rather than WNT signaling (Bao et al., 2015). Although the results are not univocal, the majority of studies show that interruption of LRP5/6 signaling has beneficial effects on infarct healing.

sFRPs

Interventions at the level of the sFRPs has been studied quite extensively by diverse research groups. Barandon et al. studied the effect of overexpression of sFRP1 on infarct healing in the mouse. Mice overexpressing the sFRP1 analogue FrzA under the control of the cytomegalovirus promoter showed smaller infarcts after either LAD occlusion or cryoinjury. This was accompanied by improved cardiac function at 15 days post-MI, less apoptosis and leucocyte infiltration in the first week after MI and a 3-fold increase in capillary density in the scar area (Barandon et al., 2003). In a follow-up study, it was shown that the infiltration of neutrophils in the infarct area could be reduced when sFRP1-overexpressing bone-marrow cells were transplanted into wild-type recipients, thereby switching the balance towards anti-inflammatory cytokine production (Barandon et al., 2011).

Regarding the role of sFRP2 in infarct healing, contradictory results have been published. sFRP2 was shown to be a key paracrine factor produced by stem cells injected around the infarct area, resulting in improved myocardial survival and repair after MI (Mirotsoy et al., 2007). Overexpression of sFRP2 in mesenchymal stem cells, injected around the infarct area, further enhanced the beneficial effect on infarct healing and cardiac function in infarcted mice (Alfaro et al., 2008). When injected directly into the border zone of the infarcted rat heart, sFRP2 reduced left ventricular fibrosis, prevented anterior wall thinning and improved cardiac function via inhibition of bone morphogenetic protein/Tolloid-like metalloproteinase (He et al., 2010). In contrast, fibrosis was found to be markedly reduced in sFRP2-null mice subjected to MI (Kobayashi et al., 2009). Moreover, sFRP2 induced marked proliferation of cardiac fibroblasts (Lin et al., 2016) and in the failing hamster heart, this pro-fibrotic response could be counteracted by treatment with a sFRP2 antibody, which reduced myocardial fibrosis and improved function (Mastri et al., 2014). At present, it is unclear how these inconsistent results can be explained.

Interventions in destruction complex

The β -catenin destruction complex consists of multiple partners, of which GSK3 β and CK1 are involved in the phosphorylation of β -catenin, the first step in the degradation of this second messenger. The potential role of GSK3 β in cardiac regeneration has been investigated both by genetic and pharmacological interventions. Because the global GSK3 β knockout is lethal, Woulfe et al. tested the effect of a tamoxifen-inducible CM-specific GSK3 β knockout on the wound healing after MI. Administration of tamoxifen from 3-8 days post-MI did not reduce infarct size, but improved cardiac function at 8 weeks post-MI and more than doubled the BrdU incorporation in CMs in the border zone (Woulfe et al., 2010). Administration of the GSK3 β -inhibitor BIO to infarcted rats for 2 weeks resulted in reduced ventricular fibrosis and improved cardiac function, but changes in infarct size or stimulation of CM proliferation were not reported in this study (Kim et al., 2016). Recently, Badimon et al. reported a small but significant decrease in infarct size mice treated with the GSK3 inhibitor SB415286, however, this effect was only observed in an ischemia-reperfusion model and not after permanent coronary ligation (Badimon et al., 2019). In this context, it has to be noted that GSK3 β -signaling in the heart is under the control of multiple other signaling pathways, as recently reviewed by Singh et al. (Singh et al., 2019) and illustrated in Fig. 2.

The effect of inhibition of CK1 with the FDA-approved anti-helminthic compound pyriminidyl on infarct healing was addressed in two studies. Saraswati et al. (Saraswati et al., 2010) reported that a single intramyocardial injection of this compound resulted in a decreased expansion of the diastolic diameter of the left ventricle, despite similar infarct size, and no change in fractional shortening. Moreover, increased numbers of Ki67⁺ myocytes were observed in the border zone and remote areas. However, since intracardiac pyriminidyl administration was found to be lethal in more than 50% of the treated mice in this study, alternative administration routes had to be explored for this compound. Intragastric administration of pyriminidyl in a mouse model of MI from day 1-14 post-MI resulted in less dilatation, fewer myofibroblasts and less fibrosis in the infarct area. Furthermore, this intervention resulted in improved cardiac function compared to control-treatment. Inhibition of WNT signaling by administering the compound SEN195 (a stabilizer of axin, the main scaffold protein in the destruction complex) for 4 weeks reduced the dilatation post-MI and improved ejection fraction in the infarcted rat heart when compared to vehicle treatment, with concomitant inhibition of WNT/ β -catenin signaling in the border zone of the infarct (Matteucci et al., 2016). Similar results were obtained in the zebrafish model of

apical resection, where endocardial Notch inhibition resulted in the release of the WNT inhibitors Wif1 and Notum1b. In this study, application of the tankyrase inhibitor IWR-1 (an axin2 stabilizer) stimulated CM proliferation and cardiac regeneration. On the contrary, activation of WNT/ β -catenin signaling with BIO inhibited CM proliferation and induced scarring (Zhao et al., 2019). Although in most of the studies in which the destruction complex was targeted, CM proliferation was not specifically investigated, the reduction in infarct size and/or improved function, observed in most of these studies, suggests that this intervention is beneficial for cardiac regeneration.

Nuclear β -catenin signaling

Direct interventions at the level of nuclear β -catenin have yielded mixed results on infarct healing. Injection of an adenovirus expressing a constitutively active form of β -catenin in the border zone of the rat infarct was reported to reduce infarct size by cell cycle activation in both CMs and fibroblasts. In the CMs, the cell cycle was not completed, giving rise to binucleation and hypertrophy. The attenuation of CM apoptosis may have contributed to the beneficial effect on infarct healing (Hahn et al., 2006), although a similar anti-apoptotic effect was observed after CM-specific deletion of β -catenin (Wo et al., 2016). CM-specific deletion of β -catenin was also reported to be beneficial by improving left ventricular function and CM survival, whereas stabilization had the opposite effect. In this study, a layer of small cells expressing cardiac Troponin-T and other cardiac lineage markers was observed at the subepicardial and subendocardial borders of the infarct, suggestive for CM proliferation (Zelarayan et al., 2008). A similar observation of cells expressing CM markers at the epicardial side was reported by treating female rats with ICG-001, a compound blocking the binding of β -catenin to the transcription complex (Sasaki et al., 2013). Recently, targeting WNT/ β -catenin at the level of the transcription complex was also demonstrated to increase the newly formed CMs and reduce scarring in the zebrafish model of apical amputation (Xie et al., 2019). Taken together, the majority of studies now shows that targeting WNT/ β -catenin at the level of the transcription complex by either a genetic or a pharmacological intervention induces a proliferative response in cells showing expression of CM markers, located at the epi-and endocardial borders of the infarct.

Discrepancy between *in vitro* and *in vivo* results regarding the modulation of WNT signaling

Overseeing the many studies published to date regarding modulation of WNT/ β -catenin signaling to induce CM proliferation reveals a rather striking dichotomy: in most of the *in vitro* studies as well as the *in vivo* studies where CMs are specifically targeted (e.g. by using a CM-specific promotor to drive a transgenic or knockout construct), activation of WNT signaling stimulates the cell cycle of CMs and leads to their proliferation. In contrast, most of the *in vivo* studies where WNT signaling is targeted with pharmacological tools, delivered either systemically or by local injection, showed that inhibition of the pathway results in smaller infarcts, and improved cardiac function. An increased proliferation of resident CMs in the border of the infarct area has also been reported in some of these studies. How can this apparent discrepancy be explained? Although this synopsis is generalized and there are certainly exceptions, it may imply that activation of WNT signaling –and more particular β -catenin-mediated WNT signaling- in CMs is sufficient to induce their proliferation. However, this mechanism might be relatively unimportant in inducing CM proliferation and cardiac regeneration after MI in an *in vivo* setting. As indicated above, infarct healing is a complex interplay between inflammatory cells, vascular endothelial cells and (myo)fibroblasts and the epi- and endocardium. Many of the mechanisms involved in the wound healing response (inflammation, angiogenesis, fibrosis, re-innervation etc.) have been shown to respond to interventions in WNT signaling as well, as reviewed elsewhere (Dawson et al., 2013; Foulquier et al., 2018). It is quite conceivable that in the plethora of signaling taking place in the healing infarct, inhibition of WNT signaling may tip the balance towards conditions where other pathways become active to stimulate CM proliferation. Moreover, activated WNT/ β -catenin signaling in CMs is associated with a hypertrophic phenotype *in vivo*, characterized by polyploidy/multinucleation and extensively developed contractile apparatus (Haybar et al., 2019). This phenotype could in fact hamper the completion of the cell cycle with cytokinesis, as discussed before. From a therapeutic point of view, a reduction in infarct size combined with CM proliferation and a better preserved pump function are essential to prevent heart failure development after MI. Since this is what pharmacological inhibition of WNT signaling in *in vivo* MI models appears to offer, we urgently need to unravel the underlying mechanisms responsible for this beneficial effect.

Follistatin-like 1

The epicardium is increasingly recognized as an essential structure in infarct healing, as it can serve as a source for secreted factors and cells that essential for cardiac repair (Cao and Poss, 2018). Wei

et al. tested the delivery of epicardial cell-derived conditioned medium to the infarcted mouse and swine hearts using a collagen patch. They observed a robust decrease in infarct size and preserved pump function, combined with the induction of CM proliferation in the border zone of the infarct. Systematic analysis of the pro-proliferative factors present in the conditioned medium resulted in the identification of Follistatin-like 1 (FSTL1) (Wei et al., 2015). Infusion or adenovirus-mediated overexpression of FSTL1 was already reported previously to have cardioprotective effects after MI (Ogura et al., 2012; Oshima et al., 2008). The post-translational glycosylation of FSTL1 at position 180 appears to be critical for its effect, as unglycosylated FSTL1, produced by the epicardium, can induce CM proliferation and cardiac regeneration, whereas the glycosylated variant, produced by the myocardium, has cardioprotective and anti-apoptotic effects (Magadum et al., 2018). A reduced infarct size combined with an improved cardiac function was recently reported upon injection of FSTL1-overexpressing mesenchymal stem cells around the infarct area, although no information was provided on the glycosylation status of the overexpressed FSTL1 (Shen et al., 2019).

Growth hormone/Insulin-like growth factor

Growth hormone (GH) and insulin-like growth factor (IGF-1) are hormones involved in regulation of the normal growth of multiple organs, including the heart. Several lines of evidence indicate that the GH-IGF-1 axis can induce reactivation of the cell cycle in adult CMs. Administration of GH to female Wistar rats for 8 weeks increased the number of CMs by 33% and increased the IGF-1 content more than 7-fold (Bruehl et al., 2007). The importance of IGF signaling in this context was demonstrated by studying zebrafish with a dominant negative form of the IGF1 receptor. This interruption in IGF signaling negatively affected CM proliferation during heart development but also during the regeneration after apical resection (Huang et al., 2013). A reduced infarct size and improved cardiac function could be demonstrated in rats when GH, embedded in alginate hydrogel, was injected in the border zone of the infarcted rat heart (Daskalopoulos et al., 2015). Interestingly, the combined local administration of IGF1 with the GSK3 β inhibitor BIO (please refer to the paragraph on WNT signaling for details) showed improved cardiac function by promoting revascularization and CM proliferation around the infarct area (Fang et al., 2015). These results show that activation of the GH/IGF1 axis promotes cardiac regeneration after MI.

Hippo

The Hippo signal transduction pathway plays an essential role in the control of organ size during development. The pathway was originally identified in *Drosophila*, but turned out to be functionally conserved over multiple species including mammals. The pathway makes use of the transcriptional co-activators YAP (Yes-associated protein) and TAZ (Transcriptional co-activator with PDX-binding motif). These co-activators can migrate to the nucleus where they can associate with transcription factors from the TEAD family and promote the transcription of proliferation-stimulating genes. Upon activation of the pathway, a cluster of kinases, including Lats1/2, Mst1/2 and Sav1, phosphorylate YAP and TAZ, resulting in their ubiquitination and destruction. Therefore, activation of Hippo signaling results in inhibition of the actions of YAP and TAZ on gene transcription and a subsequent attenuation of cell proliferation. Many proteins have been identified to regulate the activity of the Hippo pathway, but the precise mechanism of activation of the signaling pathway is unclear (Zhao et al., 2010). Further research on Hippo-YAP/TAZ signaling has revealed its importance in cancer development and tissue regeneration in adult organisms (Wang et al., 2018). Inactivation of Hippo pathway components resulted in enlarged hearts with increased CM proliferation not only in mouse embryos (Heallen et al., 2011), but also in adult mice (Heallen et al., 2013). CM-specific deletion of YAP resulted in progressive dilation and heart failure in adult mice and shifted the regenerative response upon cardiac injury towards fibrosis in newborn mice, whereas overexpression of a constitutively active form of YAP had a pro-regenerative effect (Xin et al., 2013). Genome-wide identification of YAP-bound loci identified *Pik3cb*, a catalytic subunit of phosphoinositol-3-kinase (PI3K), as a YAP effector molecule, linking Hippo signaling to the PI3K/Akt pathway to regulate CM proliferation and survival (Lin et al., 2014). Inhibition of Hippo signaling by conditionally deleting the pathway component Salv at 3 weeks post-MI, followed by a 6-week follow-up, resulted in less fibrosis, a larger CM area and an improved function compared to wildtype controls (Leach et al., 2017). Taken together, these studies support an inhibitory role for Hippo signaling on CM proliferation and cardiac regeneration in newborn and adult mice (Wang et al., 2018). Recently, a novel drug named TT-10 was described which enhances YAP-TAZ activity, resulting in increased proliferation of murine CMs *in vitro* (Ito et al., 2019). Intraperitoneal injection of TT10 in infarcted mice reduced infarct size, improved cardiac function and increase the numbers of PHH3- and Aurora B-positive CMs in the border zone (Hara et al., 2018). These results

provide a perspective for pharmacological rather than genetic intervention in Hippo signaling to stimulate CM proliferation in injured hearts.

Neuregulin-1

Neuregulin-1 (NRG1) is a member of the neuregulin growth factor family, and is the isoform most present in the heart, where the tyrosine kinase receptors ErbB4 and –to a lesser extent- ErbB3 serve as its receptors (Rupert and Coulombe, 2015). Upon binding, ErbB receptors can form a heterodimer with ErbB2 and activate cardioprotective signaling pathways (Vermeulen et al., 2016). The importance of the ErbB2 protein in cardiac physiology was highlighted in studies where cardiotoxicity was reported in breast cancer patients treated with trastuzumab, a recombinant monoclonal antibody directed against ErbB2. These adverse cardiac events were particularly apparent in patients who received trastuzumab in combination with other chemotherapeutics such as anthracyclins (Slamon et al., 2001), suggesting an impaired capacity of the heart to repair damage.

NRG1/ErbB signaling in cardiac regeneration and CM proliferation has been studied extensively in zebrafish. Upon cardiac injury, NRG1 expression peaked to more than ten-fold of the control values at 7 days after injury; the expression appeared to be localized in the outer layers of the ventricle.

Pharmacological inhibition of ErbB receptors with the inhibitor AG1478 decreased CM proliferation by ~50%. On the other hand, overexpression of NRG1 in uninjured hearts induced CM dedifferentiation and subsequent proliferation, resulting in cardiomegaly. These findings support a stimulating role for NRG1 on CM proliferation in zebrafish. Moreover, NRG1 overexpression regulated recruitment of non-CMs and blood vessel formation in the area of injury, imperative for successful cardiac regeneration (Gemberling et al., 2015). Evidence for a similar role of NRG1/ErbB signaling in mammalian CM proliferation was collected by studying ErbB2 expression in neonatal mouse hearts. A marked reduction in ErbB2 expression in the first week after birth was paralleled by a loss of CM proliferation. Moreover, conditional ErbB2 ablation in the neonatal mouse heart attenuated cell cycle activation and CM proliferation in the first week after birth (D'Uva et al., 2015; Ma et al., 2016).

Activation of NRG/ErbB signaling in mammalian models of cardiac remodeling resulted in an improved left ventricular structure and function with a concomitant reduction in CM apoptosis, increased angiogenesis and reduced cardiac fibrosis (Vermeulen et al., 2016). These positive results formed the basis for several clinical trials. Administration of a 61 aa peptide covering the epidermal growth factor domain of human NRG-1 (rhNRG-1) to human heart failure patients resulted in reduced end-systolic and end-diastolic volumes and improved acute and long-term hemodynamic effects, although a clear dose-response relationship was lacking (Gao et al., 2010; Jabbour et al., 2011). A sustained and

dose-dependent improvement of the left ventricular ejection fraction was observed in a study by Lenihan, in which ascending doses of the full-length recombinant NRG-1 β 3, also known as cimaglerin alfa, was administered to heart failure patients (Lenihan et al., 2016). However, it has to be noted that –in contrast to the animal studies- no results have been published from studies where NRG-1 treatment is tested in acute MI patients.

Because of its interaction with different members of the ErbB family, NRG1 can activate multiple signaling pathways. As mentioned above, CMs contain ErbB2, -3 and -4 receptors, the latter two being able to interact with NRG1. For ErbB signaling, dimerization is required; this can either be homo-dimerization or hetero-dimerization of e.g. ErbB2 and ErbB4. Heterodimers of ErbB2 and ErbB3/4 can activate ERK signaling, whereas ErbB4 homodimers can activate the Akt pathway. In both cases, this stimulates CM growth, proliferation and survival. Using bivalent NRG1, the formation of ErbB4 homodimers could be promoted and this was sufficient for the cardioprotective effect. Experiments performed by D'Uva et al. suggest that CM dedifferentiation and proliferation were ERK-dependent, whereas CM hypertrophy and survival could be promoted by both Akt- and ERK-activation (D'Uva et al., 2015).

Notch

Notch signaling is a highly conserved pathway involved in cell-cell communication during development and in adult life. In mammals, the pathway consists of the Notch family of receptors (Notch 1-4), single-pass transmembrane proteins with a large extracellular domain. The ligands for this receptor family are also single-pass transmembrane proteins; in mammals, they are named Jagged-1 and -2 and Delta-1, -3 and -4. Activation of the pathway requires close contact of two neighboring cells, one expressing the ligand and the other the receptor. Upon this interaction, the Notch protein is cleaved by TACE (extracellularly), and γ -secretase (intracellularly) and the intracellular domain of the Notch receptor travels to the nucleus where it can interact with the transcription repressor RBPJK to activate transcription of specific genes such as master mind-like (Maml) (Luxan et al., 2016).

Manipulation of Notch signaling during development can give rise to a plethora of cardiovascular phenotypes; for an overview we refer to a review article by Gude et al. (Gude and Sussman, 2012). Notch signaling was reported to be inactive in adult mouse hearts, but activation of Notch signaling in the border zone of the infarct area reduced infarct size, improved cardiac function and activated

proliferative signaling in mice (Gude et al., 2008). Interestingly, the cardiac regeneration observed in zebrafish was also shown to be dependent on Notch signaling, because inhibition of the pathway resulted in scar formation rather than cardiac regeneration upon amputation of the ventricular apex (Zhao et al., 2014). In a follow-up study from the same research group, it was shown that Notch receptors are expressed in epi- and endocardium rather than in myocardium and that Notch-mediated CM proliferation was dependent on the transcription of the WNT inhibitors *Wif1* and *Notum1b* in endocardial cells (Zhao et al., 2019). These results support the importance of crosstalk between different cell types in the infarcted heart.

Crosstalk between signaling pathways to promote cardiomyocyte proliferation

In a recent study, Mohamed et al. (Mohamed et al., 2018) reported that overexpression of the cell cycle proteins cyclin B1 and D1, together with cyclin-dependent kinase-1 and -4 was sufficient to induce proliferation in 15-20% of post-mitotic CMs obtained from different species. This study supports the idea that the variety of signaling pathways, discussed in the previous paragraphs of this review, eventually have to converge into a limited number of nodal points controlling the cell cycle in CMs, as recently reviewed by Hashmi and Ahmad (Hashmi and Ahmad, 2019). In Fig. 2, a simplified overview of the main signaling pathways is presented; most of the signaling pathways appear to interact with the PI3K/AKT/GSK3 β axis. In some studies, this crosstalk between the different signaling pathways was specifically addressed. The CM proliferation, observed in neonatal mice in which Hippo signaling was inactivated, resulted in increased nuclear β -catenin accumulation and could be corrected by deleting a single β -catenin allele in the CMs (Heallen et al., 2011). Along similar lines, the activation of endocardial Notch signaling in zebrafish hearts upon injury was shown to induce the release of two inhibitors of WNT signaling, Wif1 and Notum1b. Administration of the WNT inhibitor IWR-1-endo could partially restore CM proliferation in hearts with defective endocardial Notch signaling, illustrating the crosstalk between Notch and WNT/ β -catenin signaling in the regenerating zebrafish heart (Zhao et al., 2019). From these examples, it becomes apparent that studying signaling pathways in an isolated, one-dimensional fashion is insufficient for the detection of critical nodal points in the control of CM proliferation.

At present, five major signaling pathways appear to be involved in the control of CM proliferation: (1) Hippo-YAP/TAZ signaling, (2) WNT/ β -catenin signaling, (3) Ras-Raf-p38 MAPK signaling, (4) PI3K-Akt signaling and (5) Jak/Stat signaling. These pathways and some points of crosstalk between them are illustrated in Fig. 2, together with the mediators that control the activity of the pathways. Recently, an overview of the effects of these pathways on the different phases of the cell cycle was published by Hashmi and Ahmad (Hashmi and Ahmad, 2019). In their model, cyclin D activity is under the control of Hippo, Wnt/ β -catenin and PI3K-Akt signaling, suggesting the involvement of these pathways in the initiation of the cell cycle. On the other hand, cyclin A and B, which stimulate entry into the G2 and mitosis phase, are negatively regulated by p38MAPK. This could imply that interventions aiming at inhibition of the pathway promote the completion of the cell cycle by cytokinesis rather than by binucleation or polyploidy.

The molecular mechanisms controlling the processes of degradation of the contractile apparatus and cytokinesis, both essential for a complete proliferative cycle of a CM yielding two daughter cells, are less well understood. Oncostatin M was reported to induce sarcomere disassembly via ERK1/2 activation whereas IGF1 can reverse this process (Szibor et al., 2014; Yuan and Braun, 2017). Interestingly, a β -catenin-independent WNT signaling pathway involving WNT5a was reported to stimulate cytokinesis by stabilizing microtubules at the midbody. Inactivation of WNT5a signaling was shown to induce multinucleation (Fumoto et al., 2012), a phenomenon that is very common in CM.

Conclusions

Extensive research efforts in the last decade have taught us that proliferation of adult CMs in mammalian hearts actually exists and can be manipulated, as illustrated in Fig. 1. This opens up potential new avenues for cardiac regeneration upon injury of the heart. Further exploration of the signaling pathways involved in the control of this exciting process are urgently needed to shift the therapy of heart failure from treating the symptoms ('care') to an actual repair of the problem ('cure'). However, from the overview of the literature presented in this review it will be clear that we have just scratched the surface of the complex signaling involved in the control of CM proliferation. Before rushing towards clinical trials, it is mandatory to expand our knowledge on the molecular control of CM proliferation. This is of particular importance in the context of the infarcted heart, where the relevance of crosstalk between CMs and other cells and structures has become apparent. To achieve this, the following aspects should be addressed:

1. It is quite obvious that the multiple signaling pathways implicated in CM proliferation have to converge at critical nodal points to activate the different stages of CM proliferation in the correct order. In most of the studies cited in this review, however, signaling pathways are approached in a rather one-dimensional fashion. Now that the evidence is mounting for extensive crosstalk between the different pathways, we have to move towards experimental approaches where the signaling is studied in a more integrated fashion.
2. Most of the *in vitro* studies included in this review make use of cultures of CMs, typically grown in a two-dimensional layer on the rigid surface of a cell culture plate. Particularly for ventricular CMs, this is a very distant reflection of their normal habitat, where they are embedded in extracellular matrix, in close contact with fibroblasts and capillaries and rhythmically paced. Moreover, either neonatal rat ventricular CMs or CMs derived from stem cells may not sufficiently reflect the characteristics of an adult CM, stressed by low oxygen supply as will be the case in the border zone of the infarct. This problem is nicely illustrated by the conflicting results regarding the manipulation of WNT signaling, obtained *in vitro* (stimulation induces cultured CM proliferation) vs. *in vivo* (inhibition of WNT signaling promotes CM proliferation in the border zone of the infarct and cardiac regeneration). As discussed in the chapter on WNT signaling, interaction of the CMs with the many cell types implicated in infarct healing (inflammatory cells, vascular endothelial cells, fibroblasts, epi/endocardial epithelium, nerve cells) apparently gives rise to an environment where this pathway is activated too strongly for CMs to

complete the cell cycle and may in fact progress to a hypertrophic phenotype. Although it will be challenging from an experimental point of view, these discrepancies need to be solved to acquire sufficient understanding of the underlying mechanisms to move towards clinical application of the findings.

3. In most studies, CM proliferation is approached with a strong focus on the induction of the cell cycle *per se*. This appears to be an oversimplification, since complete CM proliferation including cytokinesis will require extensive degradation of the sarcomeric structure (Szibor et al., 2014). At present, little is known about this process, other than that stimuli such as Oncostatin M can induce the CM dedifferentiation required for cytokinesis (Poling et al., 2012). Interestingly, dedifferentiation can also be observed in CMs in the border zone of the infarct area. This process may resemble CM hibernation, a term defined several decades ago, describing changes in contractile machinery and cell metabolism in CMs under stress (Szibor et al., 2014). Insufficient dedifferentiation may actually impair cytokinesis (Jopling et al., 2010), driving the CM towards polyploidy, multinucleation and the hypertrophic phenotype that is frequently observed in injured hearts. On the other hand, redifferentiation of the daughter cells will also be required after successful division of a CM (Szibor et al., 2014). This has to be accompanied by appropriate electromechanical coupling to the cellular environment (Ali et al., 2014), to make the daughter cells contribute efficiently to the cardiac contraction and avoid arrhythmias. This may mean that interventions have to be carefully timed during the wound healing following MI in order to address the different phases of the CM proliferation with the appropriate instruments.

Authorship contributions

Participated in research design: Blankesteyn

Performed data analysis: Blankesteyn

Wrote or contributed to the writing of the manuscript: Blankesteyn

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Figure Legends

Fig. 1: Schematic representation of the different responses to cardiac injury: In newborn mammals and zebrafish, complete regeneration of the cardiac tissue (purple) has been reported, whereas the wound healing response in adult mammals typically yields scar tissue (blue). The pro-regenerative interventions, described in this review, typically aim at shifting the balance from scar formation towards cardiac regeneration. This result in smaller infarcts and less dilatation of the left ventricle. The black arrow heads point at regions in the infarct where cardiomyocytes were detected, which may reflect the induction of cardiac regeneration by pro-regenerative interventions. Please note that the histological sections used to illustrate cardiac regeneration are taken from adult mouse hearts.

Fig. 2: Overview of the main signaling pathways involved in cardiomyocyte proliferation. Most of the pathways activate the expression of genes involved in the initiation of the cell cycle (G1/S phase) whereas p38-MAPK is more associated with the regulation of the expression of genes associated with the late phase of the cell cycle (G2/M phase) (Hashmi and Ahmad, 2019). Extensive crosstalk has been reported between the different signaling pathways, as indicated by the green and red lines, as described in the text. Please note that most of the signaling pathways appear to be connected, either directly or indirectly, to the PI3K/AKT/GSK3 β axis. For an explanation of the abbreviations, please refer to the list of non-standard abbreviations provided with this manuscript.

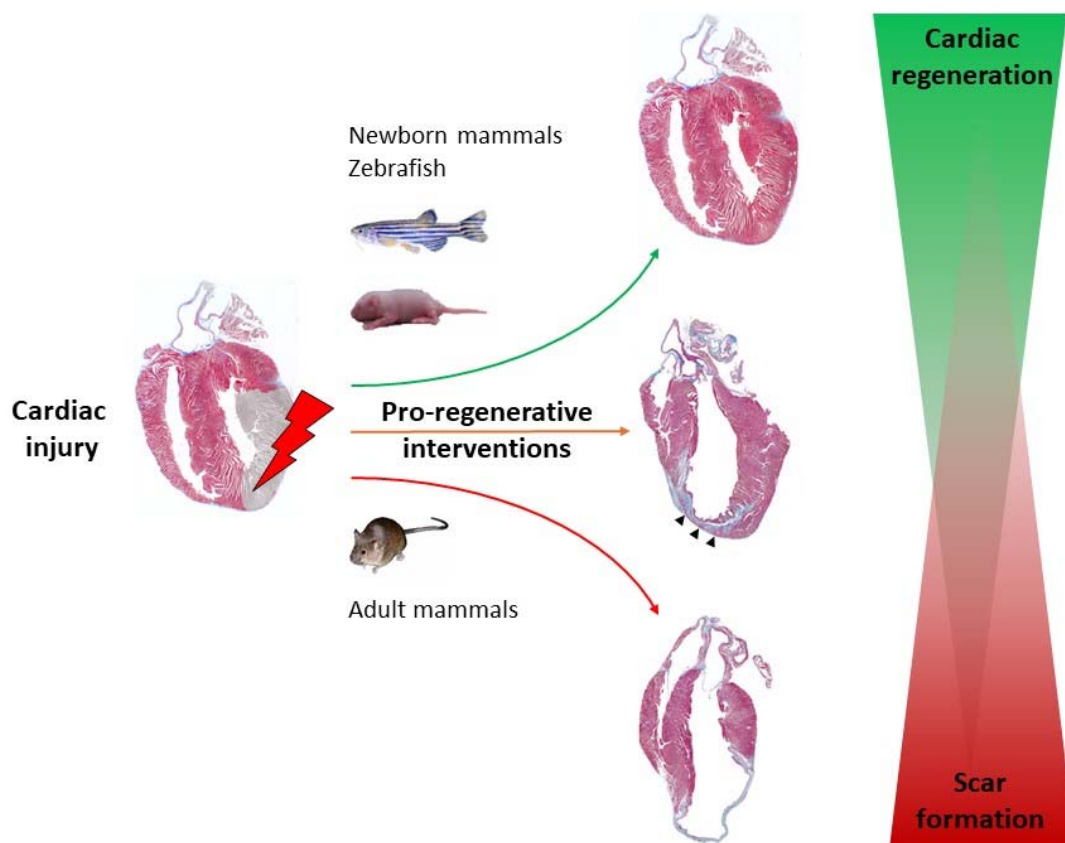


Figure 1

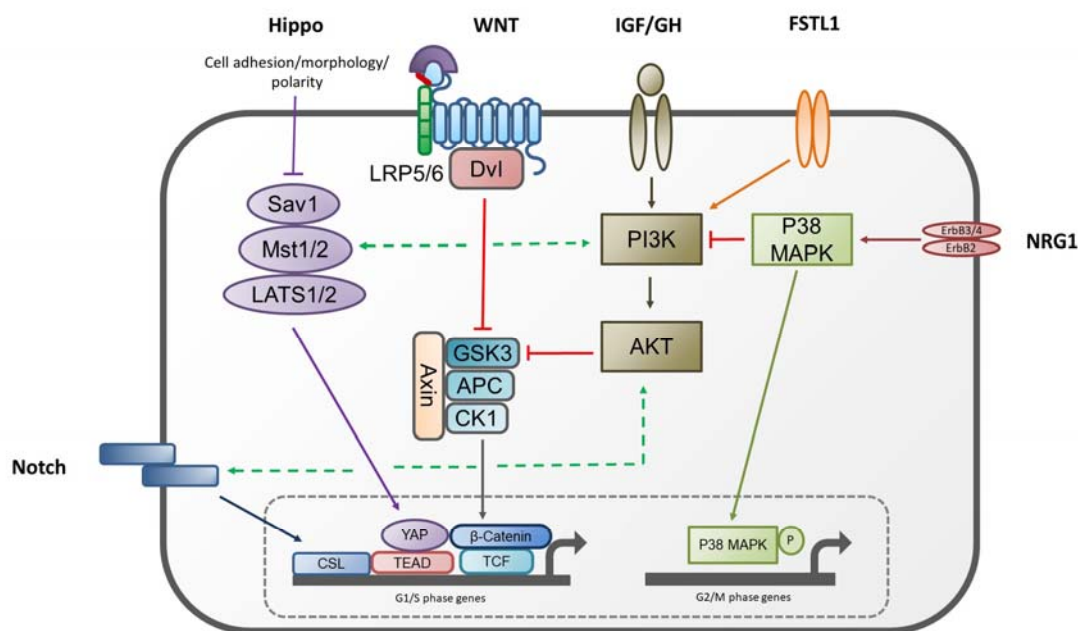


Figure 2